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Anti-inflammatory and *in vitro* Antioxidant Property of *Trigonella foenum graecum* Seeds

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ABSTRACT

Trigonella foenum graecum (Leguminosae) (Eng: fenugreek, Tamil: Vendayam) is a well known spicy agent and seeds are used traditionally for several medicinal purposes. In present study the ethanol extract of seeds from *Trigonella foenum graecum* (EETFG) was evaluated for its anti- inflammatory activity in acute (carrageenan, histamine and serotonin induced rat paw oedema) and chronic models (cotton pellet induced granuloma). In all models the EETFG (75 and 150 mg kg^{-1} b.wt. p.o.) exhibited significant anti-inflammatory activity ($p<0.001$) in a dose dependent manner when compared with saline control. Indomethacin (10 mg kg^{-1} b.wt. p.o.) was used as reference drug. The EETFG was evaluated for its antioxidant properties by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and *in vitro* lipid peroxidation induced by the Fe^{2+}-ascorbate system in rat liver homogenate. In DPPH radical scavenging assay, the EETFG demonstrated marked and dose dependent free radical scavenging effect and the mean inhibitory concentration (IC_{50}) of the EETFG was found to be 75.2 μg mL^{-1} while the ascorbic acid (reference) exhibited 43.7 μg mL^{-1}. The EETFG effectively inhibited the lipid peroxidation in a dose related manner showing the IC_{50} value of 279.1 μg mL^{-1}, whereas the quercetin (reference) showed 46.6 μg mL^{-1}. These findings revealed that the *Trigonella foenum graecum* seeds had remarkable acute and chronic anti-inflammatory and *in vitro* antioxidant actions in the tested models validating its traditional uses.

Key words: *Trigonella foenum graecum*, anti-inflammatory, carrageenan-induced paw oedema, cotton pellet-induced granuloma, radical scavenging, lipid peroxidation

INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals (Benzie, 2003). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Fang *et al*., 2002; Manavalan and Ramasamy, 2001). The most common Reactive Oxygen Spices (ROS) include super oxide anion (O_2.), hydroxyl radical (OH.), hydrogen peroxide (H_2O_2) peroxyl radical radicals (ROO.). The nitrogen derived free radicals are nitric oxide (NO.) and peroxynitrite anion (ONOO.) (Nagendrappa, 2005). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and cardio vascular malfunction (Nordberg and Arner, 2001; Ray and Husain, 2002). In treatments of these diseases, antioxidant

therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS; any may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals and also by acting as oxygen scavengers (Halliwell and Gutteridge, 1999; Daniel *et al*., 1998). Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability (Agarwal and Prabakaran, 2005; Chaurasia *et al*., 1995). Poly phenol compounds such as flavonoids and phenolic groups widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti inflammatory, anti tumor etc. (Irshad and Chaudhuri, 2002; Huang *et al*., 2005). They were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties (Lee *et al*., 2004).

In Indian system of medicine *Trigonella foenum graecum* is an important medicinal plant and its leaves and a seed has been used in various ailments and as health tonic. *Trigonella foenum graecum* (leguminosae) (Eng: Fenugreek, Tamil: Vendayam) is a well known spicy agent which prevent ageing, labour pain, impart immunity, improve mental function and add vitality to the body and it is also used in nervous disorders, dyspepsia, tumors, cholesterolemic, hyperglycemic and ulcer (Nadkarni, 1954). Reports indicate that the pharmacological activities of *Trigonella foenum graecum* include anti diabetic, antifertility, antifungal, analgesic, antipyretic and immunomodulatory activities (Bin-Hafeez *et al*., 2003; Ahmadiani *et al*., 2001). EETFG contains alkaloids flavonoids, saponins, carbohydrates, proteins and tannins. Therefore, the objectives of the present study were to investigate anti-inflammatory investigations carried out on *Trigonella foenum graecum* of Indian habitat. The present work therefore, attempts to report the preliminary results of studies on anti-inflammatory and *in vitro* antioxidant effects of *Trigonella foenum graecum* seed in experimental models to justify the traditional and folkloric beliefs.

MATERIALS AND METHODS

The seeds of *Trigonella foenum graecum* were collected from Coimbatore district, Coimbatore, Tamilnadu, India. The plant material were identified and authenticated by Dr. R. Gopalan, Director, Botanical Survey of India, Tamilnadu Agriculture University and Coimbatore, India at January 2007 (Ref. No.BSI/SC/5/23/06-07/Tech-304). The Voucher Specimen is available in the herbarium file of our department.

Preparation of the extract: The dried seed were pulverized into fine powder using a grinder and sieved through No.22 mesh sieve and stored in an air tight container. About 750 mL of 70% ethanol was added to 75 g of powder and kept on a mechanical shaker for 72 h, the content was filtered and concentration under reduced pressure, under controlled temperature of 40°C, to yield a dark oily residue. The concentrated extract was stored dry at 4°C in amber colored jars with Teflon lined caps. The percentage yield of the *Trigonella foenum graecum* ethanolic extract was found to be 4.1% w/v.

Drugs and chemicals: λ-Carrageenan (type IV) was obtained from S.D. Fine Chemicals Ltd., Bombay; 5-hydroxytryptamine hydrochloride (serotonin), histamine sulphate, thiobarbituric acid

were from Sigma Chemical Co., USA; indomethacin was from Recon, Bangalore, India; 2,2 Diphenyl-1-picryl hydrazyl hydrate (DPPH), linoleic acid, ammonium molybdate, β-carotene were purchased from Himedia, Mumbai, 2deoxy 2-ribose, xanthine oxidase, quercetin, hypoxanthine, pyrocatechol were purchased from SRL, Mumbai, thiobarbituric acid, trichloroacetic acid, Folin ciocalteu reagent, were purchased from SD Fine Ltd, Mumbai, calf thymus DNA from Genei chemicals, Bangalore, ferrozine, (2'-2' azobis (2-amidinopropane) dihydrochloride), Trolox from Sigma Aldrich, USA, 2,7 Dichloro flurescein diacetate from Fluka and Butylated hydroxyl toluene from Loba cheme. All other chemicals used in the study were of analytical grade procured from local suppliers.

Experimental animals: Wistar albino rats of either sex (150-200 g) were used for this study. They were housed in standard polypropylene cages and kept under controlled room temperature (24±20°C, relative humidity 45-55%) in a 12 h light-dark cycle. The rats were given a standard laboratory diet and water *ad libitum*. The study was conducted after ethical clearance from the institutional animal ethics committee bearing the reference number 817/04/ac/CPCSEA.

Phytochemical screening: Preliminary phytochemical screening of the powdered seed was performed for the presence of alkaloids, phenolics, flavonoids, saponins, tannins, carbohydrates and proteins.

Acute toxicity: The oral LD_{50}value of EETFG in male Swiss albino mice was determined as per reported method (Dixon, 1965; Abukakar *et al*., 2008).

Anti-inflammatory activity

Carrageenan-induced rat paw oedema: The rats were divided into four groups (n = 6). The first group (which served as control) received normal saline (0.9% w/v, 3 mg kg^{-1} b.wt., p.o.). The second and third group received the test. Extract EETFG (75 and 150 mg kg^{-1} b.wt., p.o., respectively). The fourth group (which served as reference) received indomethacin (10 mg kg^{-1} b.wt., p.o.). After 30 min, acute inflammation was produced by the subplantar administration of 0.1 mL of 1% (w/v) of freshly prepared suspension of carrageenan in the right hind paw of each rat. The paw volume was measured at 0 and 3 h after carrageenan injection by using plethysmometer (Ug Basile, Italy). The difference between the two readings was taken as the volume of oedema and the percentage of inhibition was calculated (Ghosh, 2008; Winter *et al*., 1962; Zakaria *et al*., 2006).

Mediator-induced inflammation: The paw oedema was induced in rats by sub plantar injection of 0.1 mL of freshly prepared histamine (1 mg mL^{-1}) and serotonin (1 mg mL^{-1}) solutions respectively (Suleyman *et al*., 1991; Parmar and Ghosh, 1978). Group division and treatment regime of the animals were same as the carrageenan induced rat paw oedema model and the paw oedema was measured.

Cotton pellet-induced granuloma: The animals were divided into four groups (n = 6). The rats were anaesthetized and sterile cotton pellets weighing 10±1 mg were implanted subcutaneously into both sides of the groin region of each rat. The first group (which served as control) received normal saline (0.9% w/v, 3 mg kg^{-1} b.wt., p.o.). The second and third group received the test extract EETFG (75 and 150 mg kg^{-1} b.wt., p.o., respectively). The fourth group (which served as

reference) received indomethacin (10 mg kg^{-1} b.wt., p.o.). All groups were treated in this way for seven consecutive days from the day of cotton pellet implantation (D'Arcy *et al.*, 1960; Raj *et al.*, 2006). On 8th day the animals were anaesthetized and the pellets together with the granuloma tissues were carefully removed and made free from extraneous tissues. The wet pellets were then dried in an oven at 60°C for 24 h to constant weight. Increment in the dry weight of the pellets was taken as a measure of granuloma formation (Winter and Porter, 1957).

***In vitro* antioxidant property**

Scavenging activity of DPPH: The antioxidant property of EETFG was determined on the basis of their scavenging activity of stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical (Obho, 2006; Gupta *et al.*, 2006). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of EETFG suspension in water at different concentrations (25-200 mg mL^{-1}). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using methanol as blank on UV-visible spectrophotometer Shimadzu, UV-1601. The scavenging activity was measured as the decrease in absorbance of the samples versus standard DPPH solution. Ascorbic acid was used as the reference. The results were expressed as percentage of inhibition at different concentrations and IC_{50} was determined. The IC_{50} (mean inhibitory concentration) value denotes the concentration of the sample (in μg mL^{-1}) required to scavenge 50% of the DPPH free radicals. The percentage scavenging activity was calculated by using the following formula,

$$I\% = \left[\frac{(A_c - A_s)}{Ac}\right]100$$

where, A_c is the absorbance of control reaction (containing all reagents except the test extract) and A_s is the absorbance of the sample at different concentrations. All the tests were performed in triplicate and the results averaged.

Determination of inhibition of lipid peroxidation

Tissue sample preparation: The liver of normal rat was excised and perfused *in vitro* with ice cold normal saline (0.9% w/v). The tissues were then homogenized at a concentration of 10% w/v in 1.15% w/v KCl solution and centrifuged at 1200 g at 4°C for 10 min. The supernatant was collected which was again centrifuged at 10000 g at-4°C for 10 min. The supernatant was taken and stored at-20°C for use in the study (Fujita *et al.*, 1998).

Estimation method: Lipid peroxidation induced by Fe^{2+}- ascorbate system in rat liver homogenate was estimated as thiobarbituric acid reacting substance (TBARS) by the method of Ohkawa *et al.* (1979). T he reaction mixture contained rat liver homogenate 0.1 mL in Tris-HCl buffer (40 mM pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)2$ $SO_4.7H_2O$ (0.16 mM); Sodium ascorbate(0.06 mM); and Various concentrations of EETFG in a final volume of 0.5 mL. The reaction mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 mL was removed and treated with 0.2 mL sodium dodecyl sulphate (SDS, 8.1%); 1.5 mL thiobarbituric acid (TBA, 0.8%) and 1.5 mL acetic acid (20%, pH 3.5). The total volume was made up to 4 mL with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling 1.0 mL of distilled water and 5.0 mL of n-butanol and pyridine mixture (15: 1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged

at 4000 rpm for 10 min. The upper n-butanol-pyridine layer was removed and its absorbance at 532 nm was measured by using UV-visible spectrophotometer Shimadzu, UV-1601. Inhibition of lipid peroxidation was determined by comparing the absorbance of treatments with that of the control. Quercetin was used as the reference.

The inhibitory ratio of the test sample was evaluated by the following formula:

$$\text{Percentage inhibition} = \frac{A_c - A_s}{A_c} \times 100\%$$

Statistical analysis: Except antioxidant studies the values were expressed as Mean±Standard Error of Mean (SEM). The statistical significance was determined by using the Student's't' test (23). Values of $p<0.001$ were considered as statistically significant.

RESULTS AND DISCUSSION

The present study establishes the significant anti-inflammatory activity of the ethanol extract of the seed of *Trigonella foenum graecum* (EETFG) in both acute and chronic models. Carrageenan-induced oedema has been commonly used as an experimental animal model for acute inflammation and it is believed to be a biphasic response. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine and serotonin (5-HT). The late phase is mediated by bradykinin, leukotrienes, polymorph nuclear cells and prostaglandins produced by tissue macrophages . The EETFG produced dose dependent and significant ($p<0.001$) inhibition of carrageenan-induced paw oedema after a period of 3 h (Table 1). The EETFG showed maximum inhibition of 57.11% at the dose of 150 mg kg^{-1} b.wt. after 3 h of treatment, whereas the reference drug indomethacin produced 71.71% of inhibition.

The EETFG also significantly ($p<0.001$) suppressed the inflammation produced by the mediators' viz. histamine and serotonin. It indicates that the EETFG inhibits the inflammation caused by carrageenan and mediators (Table 2 and 3). The EETFG produced 51.89 % inhibition in case of histamine and 59.66 of inhibition in case of serotonin at the dose of 150 mg kg^{-1} b.wt.; while the reference drug indomethacin produced 62.37 and 70.63% of inhibition of paw oedema, respectively in above two mediators.

The cotton pellet method is widely used to evaluate the exudative and proliferative components of the chronic inflammation. Chronic inflammation is a reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation. Chronic inflammation occurs by the development of proliferative cells. These cells can either spread or remain in granuloma form. The dry weight of the cotton pellets correlates with the amount of the granulomatous tissue formed (Olajide *et al*., 1999, 2000). The EETFG showed significant ($p<0.001$) and dose dependent anti-inflammatory action in cotton pellet induced granuloma and hence found to be effective in chronic inflammatory conditions. Based on the results it can be concluded that the EETFG possesses anti-inflammatory potential in both acute and chronic phases of inflammation (Table 4). The EETFG produced the maximum inhibition of 53.99% at the dose of 150 mg kg^{-1} b.wt. and the reference drug indomethacin produced 64.06% of inhibition of granuloma formation.

The results of the present study indicate that the EETFG has effective degrees of *in vitro* antioxidant activity by the methods employed. It is now well established that free radicals

Table 1: Effect of EETFG on carrageenan induced rat paw oedema

Treatment	Dose (mg kg^{-1})	Increase in paw volume (mL)	Percentage of inhibition
Control	-	0.774±0.13	-
EETFG	75	0.483±0.08*	43.67
EETFG	150	0.332±0.04*	57.11
Indomethacin	10	0.219±0.02*	71.71

Values are Mean±SD and significant at *p<0.001 for n = 6 where compared to control group

Table 2: Effect of EETFG on histamine induced rat paw oedema

Treatment	Dose (mg kg^{-1})	Increase in paw volume (mL)± SEM	Percentage of inhibition
Control	-	0.582±0.024	-
EETFG	75	0.341±0.008*	41.41
EETFG	150	0.280±0.015*	51.89
Indomethacin	10	0.219±0.006*	62.37

Values are Mean±SD and significant at *p<0.001 for n = 6 when compared to control group

Table 3: Effect of EETFG on serotonin induced rat paw oedema

Treatment	Dose (mg kg^{-1})	Increase in paw volume (mL)±SEM	Percentage of inhibition
Control	-	0.647±0.007	-
EETFG	75	0.389±0.011*	39.88
EETFG	150	0.261±0.005*	59.66
Indomethacin	10	0.190±0.003*	70.63

Values are Mean±SD and significant at *p<0.001 for n = 6 when compared to control group

Table 4: Effect of EETFG on cotton pouch induced granuloma in rats

Treatment	Dose (mg kg^{-1})	Increased wt of cotton pellet (mg)	Percentage of inhibition
Control	-	39.23±0.18	-
EETFG	75	25.72±0.35*	34.44
EETFG	150	18.05±0.27*	53.99
Indomethacin	10	14.10±0.38*	64.06

Values are Mean±SD and significant at *p<0.001 for n = 6 when compared to control group

(e.g., superoxide, hydroxyl radical, nitric oxide) and other reactive species (e.g., hydrogen peroxide, singlet oxygen, peroxynitrite, hypochlorous acid) contribute to the pathology of many disorders including arthritis and connective tissue disorders, ageing, neurodegeneration, chronic inflammation and cancer (Tripathi *et al*., 1996). Free radicals may also be a contributory factor in the function of the immune system (Hemnani and Parihar, 1998). Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species (which act as pro-inflammatory agents) from phagocytes invading the inflammation sites (Yoshikawa *et al*., 1983). The DPPH test provides information on the reactivity of test extract with a stable free radical. DPPH is stable nitrogen centered free radical containing an odd electron in its structure that can accept an electron or hydrogen radical to become a stable diamagnetic molecule and usually utilized for detection of radical scavenging activity. Because of its odd electron DPPH gives a strong absorption at 517 nm in the visible region (deep violet colour). As the electron becomes paired off in presence of a free radical scavenger, the absorption diminishes, thus the resulting decrease in absorbance is stoichiometric with respect to the number of electrons taken up. The EETFG exhibited marked and dose dependent free radical scavenging effect in DPPH radical scavenging assay showing the IC_{50} value 75.2 μg mL^{-1} (Table 5). The percentage of inhibition

Table 5: Antioxidant property of EETFG on DPPH radical scavenging activity

Test sample	Percentage of inhibition*	IC_{50} (μg mL^{-1})
Control	-	
EETFG + DPPH (μg mL^{-1})		
25	9.18	75.2
50	18.50	
100	38.38	
200	64.23	
Ascorbic acid (μg mL^{-1})		
50	51.33	43.7
100	67.02	

*Values are Means (n = 3)

Table 6: Antioxidant property of EETFG on Fe2+- ascorbate induced lipid peroxidation

Test sample	Percentage of inhibition*	IC_{50} (μg mL^{-1})
Control	-	-
EETFG (μg mL^{-1})		
10	28.67	279.1
100	37.08	
1000	66.98	
Quercetin (μg mL^{-1})		
10	44.17	46.6
100	51.15	

*Values are Means (n = 3)

was found to be 64.23% at the concentration of 200 μg mL^{-1} and 9.18% at the concentration of 25 g mL^{-1}. The IC_{50}value of EETFG was 75.2 μg mL^{-1}. Ascorbic acid was used as reference and its IC_{50} value was found to be 43.7 μg mL^{-1}.

Lipid peroxidation is complex process whereby polyunsaturated fatty acids of cellular membranes undergo reaction with reactive oxygen species to yield lipid hydro-peroxides. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydro- peroxides into peroxyl and alkoxyl radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). This lipid peroxidation can be prevented either by reducing the formation of free radicals or by supplying the competitive substrate for unsaturated lipids in the membrane or by accelerating the repair mechanisms of damaged cell membrane. Several natural and synthetic antioxidants are used to prevent the lipid peroxidation (Valentao *et al*., 2002).

The antioxidant activity of the EETFG was further confirmed by evaluating the inhibition in production of malondialdehyde (MDA) and related carbonyl products that are produced as by products of lipid peroxidation induced by Fe^{2+}- ascorbate system in the biomembranes of rat liver homogenate. These carbonyl products are responsible for DNA damage, carcinogenesis and aging related diseases. The MDA reacts with thiobarbituric acid in specific reaction medium to produce a strong absorption at 532 nm. The EETFG effectively inhibited the lipid peroxidation, respectively. The IC_{50} value of $_{EETFG}$ was 279.1 μg mL^{-1} (Table 6). The percentage of inhibition was 66.98 and 28.67% at the concentrations of 1000 and 10 μg mL^{-1}, respectively. The IC_{50} value of EETFG was 279.1 μg mL^{-1} and that of quercetin (reference) was found to be 46.64 μg mL^{-1}. This activity is perhaps related to the H^+ ion donating capability of the extract which can scavenge the peroxyl radical to inhibition or termination of the peroxidation chain.

Preliminary phytochemical analysis indicated the abundance of true alkaloids and flavonoids in EETFG. Its anti-inflammatory and antioxidant potential could be attributable to these putative constituents. Flavonoids are well known natural antioxidants due to their electron donating property which either scavenge the principal propagating free radicals or halt the radical chain. Thus the antioxidant activity of EETFG may be due to the presence of flavonoids which augmented the anti-inflammatory action.

CONCLUSION

Present investigation confirms significant acute and chronic anti-inflammatory and *in vitro* antioxidant properties of the EETFG in the tested models. It can be inferred that the anti-inflammatory activity of EETFG may be due to the inhibition of free radicals production that act as pro-inflammatory agents in acute and chronic inflammation. So the antioxidant property of EETFG can explain, in part, the mechanism of its anti-inflammatory activity. Present study therefore, substantiates the traditional uses of *Trigonella foenum graecum* seed in pain and rheumatism in South India. Further studies are presently underway to confirm the identity of bioactive principles responsible for these actions by the seed of *Trigonella foenum graecum*.

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